Ministry of Economic Development
Manatū Ōhanga
Intellectual Property Office of New Zealand

CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 14 June 1999 with an application for Letters Patent number 336259 made by AUCKLAND UNISERVICES LIMITED.

Dated 7 March 2006.

Neville Harris
Commissioner of Patents, Trade Marks and Designs

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336259

Patents Form No. 4

PATENTS ACT 1953

PROVISIONAL SPECIFICATION

CANCER THERAPY

We, **AUCKLAND UNISERVICES LIMITED**, a New Zealand company of 58 Symonds Street, Auckland, New Zealand, do hereby declare this invention to be described in the following statement:

CANCER THERAPY

FIELD OF THE INVENTION

This invention is directed to the use of therapeutic agents in combination to combat cancer. In particular it is directed to combinations of therapeutic agents which are effective against advanced and large tumour burdens.

BACKGROUND TO THE INVENTION

Advanced cancers and large tumour burdens are refractory to treatment with therapeutic agents. Although these same agents may be effective against smaller tumours, their use does not achieve complete eradication of large tumour burdens. Large tumours can continue to grow unchecked, or their re-growth is not recognised by the body's immune system.

In addition, tumours acquire defensive and survival functions which limit the efficacy of therapeutic agents and/or the body's own immune response. For unknown reasons large tumour burdens appear to either impair or retard the generation of anti-tumour cytotoxic T lymphocyte responses. In immunotherapy, gene transfer of T cell co-stimulatory cell adhesion molecules is effective against only very small tumours and only weak anti-tumour systemic immunity is generated.

It is an object of the present invention to provide a therapeutic combination that will at least partially overcome the resistance of large tumour burdens to immunotherapy, or at least provide the public with a useful choice in the treatment of cancer.

SUMMARY OF THE INVENTION

Accordingly, in a first aspect the invention provides a method of treatment for mammals, including humans, with advanced or large tumour burdens comprising of the administration of an immunotherapeutic agent in conjunction with a tumour growth-restricting agent, either of which alone would be ineffective in retarding or eradicating an advanced or large tumour burden.

In a further aspect, the invention provides a method of treating a patient with cancer which comprises the step of administering to said patient an immunotherapeutic agent and a tumour growth restricting agent in amounts which are together effective to eradicate any advanced or large tumours present.

In still a further aspect, the invention provides a method of potentiating the activity of an immunotherapeutic agent against tumours present in a patient suffering from cancer which comprises the step of administering to said patient when treated with said immunotherapeutic agent an amount of a tumour growth restricting agent, which is effective, in combination with the immunotherapeutic agent to eradicate any advanced or large tumours present.

In yet a further aspect, the invention provides a method of potentiating the activity of a tumour growth restricting agent against tumours present in a patient suffering from cancer which comprises the step of pre-administering to a patient to be treated with said tumour growth restricting agent an amount of an immunotherapeutic agent which, upon subsequent administration of said tumour growth restricting agent, acts in combination with said tumour growth restricting agent to eradicate any advanced or large tumours present.

As used herein, the term "immunotherapeutic agent" means a preparation containing DNA which when administered to the patient results in a systemic anti-tumour immune response. Typically the immunotherapeutic agent will be a pharmaceutically acceptable formulation of DNA to be injected into the tumour at one or more sites so as to confer properties on the tumour tissue which generate a systemic anti-tumour immune response.

As used herein, the term "tumour growth restricting agent" means an agent which restricts or prevents tumour growth in a patient through reducing blood flow to tumours, including by inhibiting or preventing angiogenesis. Such an agent may also have other anti-tumour\immunoregulatory activities in addition to reducing blood flow.

Preferably the DNA of the immunotherapeutic agent will encode a T cell co-stimulatory cell adhesion molecule (CAM), more preferably in a suitable expression

vector. Most conveniently the CAM will be B7.1, B7.2 or a xenogenic (human) form of an integrin ligand, or combinations thereof.

Conveniently, the tumour growth restricting agent is flavone acetic acid (FAA) or an analogue thereof. The FAA analogue 5,6-dimethylxanthenone-4-acetic acid (DMXAA) is particularly preferred.

Preferably, the immunotherapeutic agent is administered prior to administration of the tumour growth restricting agent. More preferably, the immunotherapeutic agent is administered from 12 to 48 hours prior to administration of the tumour growth restricting agent. Most preferably, administration of the immunotherapeutic agent occurs approximately 24 hours prior to administration of the tumour growth restricting agent.

In still a further embodiment, the present invention provides a chemotherapeutic pack which includes, in separate containers, both an immunotherapeutic agent and a tumour growth restricting agent as defined above.

In still a further aspect, the invention provides for the use of a tumour growth restricting agent in the preparation of a medicament for potentiating the activity of an immunotherapeutic agent against advanced or large tumours.

In yet a further aspect, the invention provides for the use of an immunotherapeutic agent in the preparation of a medicament for potentiating the activity of a tumour growth restricting agent against advanced or large tumours.

DESCRIPTION OF THE DRAWINGS

While the invention is broadly defined as above, those persons skilled in the art will appreciate that it is not limited thereto and that it also includes embodiments of which the following description provides examples. In addition, the present invention will be better understood from reference to the accompanying drawings in which:

Figure 1. B7.1 gene transfer is unable to cause the rejection of large tumours. Established tumours 0.5 cm in diameter were injected with DOTAP liposomes

containing 60 μg of B7.1 cDNA. Control animals received 60 μg of empty pCDM8 vector. Tumour growth was slowed by B7.1 gene transfer, but ultimately tumours grew unchecked, and animals had to be euthanased.

Figure 2. Combining the drugs DMXAA and FAA with B7.1 gene therapy leads to the complete eradication of large tumours, and the generation of potent anti-tumour systemic immunity. Established tumors approximately 0.6-0.8 cm in diameter were injected with DOTAP liposomes containing 60 μg of B7.1 cDNA. Control animals received 60 μg of empty pCDM8 vector, or liposomes alone, as indicated. Other animals bearing 0.6-0.8 cm tumours were injected i.p. with DMXAA and FAA at 300 mg/Kg and 25 mg/Kg of body weight in a volume of 0.01 ml/g body weight, respectively. For animals receiving combinational treatments, B7.1 cDNA was administered and DMXAA and FAA 24 h later. The size (cm) of tumors was monitored for 42 days following gene transfer. Mice were euthanased if tumors reached more than 1 cm in diameter (denoted by small vertical arrows). The experiment was repeated twice (n=6). Mice that were cured of their tumours were rechallenged (large vertical arrow) after 42 days with 10^6 parental tumour cells, and mice monitored for tumor regrowth for a further 22 days.

Figure 3. Comparison of anti-tumour CTL activity generated by the different treatment regimes. A) Splenocytes were removed from animals 21 days following the different treatment regimes, and were tested for cytolytic activity against EL-4 tumour cells. The percent cytotoxicity is plotted against various effector to target (E: T) ratios. Control animals received empty pCDM8 vector or liposomes alone. B) Cytolytic activity of splenocytes harvested from animals 42 days after treatment with B7.1/DMXAA, and a further 22 days later (day 64) following a rechallenge with parental EL-4 tumour cells.

DESCRIPTION OF THE INVENTION

As outlined above in broad terms, the present invention provides a method of combination therapy for the treatment of patients with advanced or heavy tumour burdens.

It has been noted that advanced tumour growth is accompanied by the ability of the tumours to acquire unknown mechanisms by which they may resist the body's

systemic anti-tumour immune response. Although the administration of chemotherapeutic agents or other means of cancer therapy may initially cause regression in the growth of the tumours, the body's immune response is unable to prevent or limit the re-growth of tumourgenic tissue that has not been eradicated from the body.

The applicants have now determined that by combining methods of immunotherapy with methods of chemotherapy previously demonstrated to be ineffective in the long term treatment of advanced or heavy tumour burdens, regression of tumours is combined with the stimulation of a strong, systemic anti-tumour immune response.

The two therapeutic agents employed therefore operate in a synergistic manner to provide a combined effect which exceeds that predictable from the known properties of each. This is particularly true where, as is presently preferred, the immunotherapeutic agent is a preparation of DNA encoding a T cell co-stimulatory cell adhesion molecule (CAM) or an integrin ligand and the tumour growth restricting agent is flavone acetic acid (FAA) or an analogue thereof.

Optimized gene transfer of T-cell co-stimulatory cell adhesion molecules (CAM), including B7.1, B7.2, and xenogeneic (human) forms of the integrin ligands VCAM-1, MAdCAM-1, and ICAM-1 has been shown to cause rapid and complete rejection of established small tumours. Prolonged systemic anti-tumour immunity is generated, whereas other cell adhesion molecules such as human E-cadherin have only a weak ability to slow tumour growth. However, CAM-mediated immunotherapy is problematic in that it is effective against only small tumours and it generates only weak anti-tumour systemic immunity. Larger tumour burdens are able to either impair or retard the generation of anti-tumour cytotoxic T lymphocytes (CTL) rendering the tumours resistant to immunotherapy.

The anticancer agent flavone acetic acid (FAA) and its analogue 5,6-dimethylxanthenone-4-acetic acid (DMXAA) cause initial reductions in tumour size when administered, but tumours subsequently grow unchecked, and both reagents generate a weak and ineffective anti-tumour CTL response. DMXAA and FAA appear to exert their anti-tumour activities via several pathways including reduction of tumour blood flow leading to hemorrhagic necrosis and the induction of multiple immunomodulatory factors including cytokines, nitric oxide, and activated natural

killer cells. However, neither agent is able to generate the desired anti-tumour systemic immunity, and they are ineffective against large tumour burdens.

The finding made by the applicants that administration of these agents in combination is effective to both eradicate advanced or large tumours and to generate anti-tumour systemic immunity is therefore surprising and representative of a significant advance in cancer treatment.

The immunotherapeutic agent can be, or include, DNA (usually cDNA) encoding human (Genbank U82483) or mouse (Genbank L21203) MAdCAM-1, human VCAM-1 (Genbank M60335), ICAM-1 (Genbank J03132), mouse (Genbank X06115) or human (Genbank L08599) E-cadherin, B7.1 (Genbank AF065896) or B7.2 (Genbank L25606). Such cDNA's can be synthesised or obtained from commercial or other sources. For example, human VCAM-1 can be obtained from R & D Systems, Abingdon, UK, human ICAM-1 can be obtained from Human Genome Sciences, Inc. (HGS), whereas B7.1 can be sourced from Dr P Linsley, Bristol-Myers-Squibb, Seattle, Washington, USA.

Sources for other cDNA's are as follows:

Human MAdCAM-1 - from HGS.

Mouse MAdCAM-1 - from Dr Eugene Butcher, Stanford University, Stanford, USA

Human E-cadherin - from Drs Rimm and Morrow, Yale University School of Medicine, New Haven, CT, USA

Mouse E-cadherin - from Dr M Takeichi, Kyoto University, Kyoto, Japan

Human B7.2 - from Dr Gordon Freeman, Dana Farber Cancer Institute, Boston, MD, USA.

The immunotherapeutic agent will generally be administered in the form of a mammalian expression vector. While any such vector available to the skilled artisan may be selected, typical vectors include expression plasmids such as

pCDNA3 and pCDM8, and adenoviral- and retroviral-based vectors (such as pLXSN and pLNCX).

The tumour growth restricting agent can be any available agent which exerts an anti-tumour effect, at least in part, by restricting tumour blood flow. The agent may also have other, equally potent, anti-tumour properties, including immunoregulatory properties.

It is presently preferred that the tumour growth restricting agent be FAA or an analogue of FAA. DMXAA is particularly preferred. However, other agents which may also be used include reagents which target the αvβ3 integrin and associated proteins, endostatin protein and cDNA, angiostatin cDNA, IL-12 cDNA, anti-sense constructs which target the VEGF's and their receptors Klk-1 and Flt-1, angiogenin, urokinase plasminogen activator (uPA) and calreticulin.

Aspects of the invention will now be described with reference to the following experimental section which is exemplary only.

EXPERIMENTAL

Materials and Methods

Mice and cell lines

Female C57BL/6 mice, 6-9 weeks old, were obtained from the Animal Resource Unit, School of Medicine and Health Science, University of Auckland, Auckland. The EL-4 thymic lymphoma and mouse Lewis lung carcinoma cells (H-2b) were purchased from the American Type Culture Collection (Rockville, MD). These cell lines were cultured *in vitro* at 37°C in DMEM medium (Gibco BRL), supplemented with 10% foetal calf serum, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM pyruvate.

FAA and DMXAA analogues

FAA was gifted from the Department of Health & Human Services, Drug Synthesis & Chemistry laboratory, National Cancer Institute, Bethesda. DMXAA was synthesized in the Cancer Research Laboratory, School of Medicine and Health Science, University of Auckland.

B7.1 pCDM8 expression vector

Complementary DNA encoding mouse B7.1 was provided by Dr P. Linsley, Bristol-Myers-Squibb, Seattle, WA, USA. pCDM8 is available from Invitrogen, USA.

Administration of drugs

Solutions of FAA and DMXAA in 5% (w/v) sodium bicarbonate and water, respectively, were prepared fresh for each experiment and protected from light. Tumours were established by subcutaneous injection of 2 x 10^5 EL-4 and Lewis lung carcinoma tumour cells into the left flank of mice, and growth determined by measuring two perpendicular diameters. Animals were euthanized when tumours reached more than 1 cm in diameter, in accord with Animal Ethics Approval (University of Auckland). EL-4 and Lewis lung tumours reached 0.6-0.9 cm in diameter after approximately 21 and 14 days, respectively. At this stage, FAA and DMXAA were injected intraperitoneally (i.p.) at 300 mg/Kg and 25 mg/Kg of body weight in a volume of 0.01 ml/g body weight, respectively. All experiments included 6 mice per treatment group, and each experiment was repeated at least once.

Gene transfer of B7.1

The B7.1 pCDM8 expression vector was prepared by cesium chloride gradient centrifugation, and diluted to 600 μg/ml in a solution of 5% glucose in 0.01% triton x-100. It was mixed in a ratio of 1:3 (wt:wt) with DOTAP cationic liposomes (Boehringer Mannheim). Tumours were injected at multiple sites with 100 μl of DNA (60 μg)/liposome complexes.

Combining B7.1 gene therapy with FAA and DMXAA

Tumours, 0.6 to 0.9 cm in diameter, were injected at multiple sites with 100 μl of DNA (60 μg)/liposome complexes. Twenty-four hours later, DMXAA and FAA were administered i.p. as described above. Treated mice that remained tumour-free were re-challenged 6 weeks after administration of FAA or DMXAA and B7-1, by s.c.

injection of 1 x 10^6 EL-4 cells and 2 x 10^5 Lewis lung carcinoma cells (0.1 ml) in the opposing flank (right flank).

Measurement of the generation of anti-tumour CTL

Splenocytes were harvested 21, and 42 days following initial gene transfer, and 22 days following a parental tumour challenge. They were incubated at 37°C with EL-4 target cells in graded E:T ratios in 96-well round-bottom plates. After a 4-hour incubation, 50 µl of supernatant was collected, and lysis was measured using the Cyto Tox 96 Assay Kit (Promega, Madison, WI). Background controls for non-specific target and effector cell lysis were included. After background subtraction, percentage of cell lysis was calculated using the formula: 100 x (experimental-spontaneous effector-target spontaneous target/maximum target-spontaneous target).

Results

B7.1 gene therapy is unable to check the growth of large tumours

EL-4 cells (2 x 10^5) subcutaneously implanted into mice grow rapidly, forming a solid tumour 1 cm in diameter within 4 weeks. We have previously demonstrated that small EL-4 tumours (0.1-0.2 cm diameter) transfected *in situ* with B7.1 cDNA failed to grow, and mice remained tumour-free for at least two months (Kanwar et al. 1999). In contrast, larger tumours (>0.3cm) are refractory to treatment, and ultimately grow unchecked (Fig. 1).

DMXAA and FAA are unable to check the growth of large tumours

Systemic administration of optimal doses of DMXAA and FAA to mice bearing large EL-4 tumours (0.6-0.8 cm in diameter) led to immediate reductions in the sizes of tumours (Fig. 2), accompanied by marked tumour necrosis. DMXAA was the more potent of the two reagents, causing tumours to shrink to 0.1-0.2 cm over a period of 3 weeks, whereas the tumours of FAA-treated animals were reduced to 0.2-0.4 cm in diameter. However, tumours began to grow unchecked by day 28 and animals had to be sacrificed during the sixth week.

DMXAA and FAA synergize with B7.1 to provide potent anti-tumour immunity

We reasoned that reagents such as DMXA and FAA, which temporarily hold tumour growth in-check may provide a critical window of time to allow CAM-mediated immunity to become effective against larger tumours. However, by impairing blood flow they would also be expected to impair the entry of anti-tumour lymphocytes into the tumour. To overcome this potential problem established tumours (0.6-0.8 cm in diameter) were first treated with B7.1 to stimulate anti-tumour immunity, and DMXAA and FAA were administered one day later to retard tumour growth. Remarkably, tumours rapidly diminished in response to the combination of B7.1 and DMXAA accompanied by massive necrosis, such that by the third week of treatment tumours had completely disappeared. Unlike B7.1 treatment, which leaves what appears to be a palpable fibrotic core, the tumour sites of DMXAA/B7.1-treated animals were completely healed. Similar results were obtained with the combination of FAA and B7.1, although tumours did not completely disappear until the sixth week.

Combined administration of FAA analogues and B7.1 gene therapy leads to enhanced tumour-specific cytolytic T cell activity

The anti-tumour CTL activity of splenocytes obtained 21 days following gene transfer was significantly ($p>0.001$) augmented in animals treated with the combination of B7.1 and DMXAA, and slightly enhanced with b7.1/FAA, versus those receiving empty vector or vehicle alone (fig. 3). Gene transfer of b7.1 alone generated a very good CTL response, whereas DMXAA and FAA alone were very poor effectors.

Combined administration of FAA analogues and B7.1 gene therapy generates potent anti-tumour systemic immunity

Animals previously cured by combination therapy completely rejected the substantial challenge of 1×10^6 parental tumour cells for the 22 days they were monitored (Fig. 2). Both DMXAA/B7.1 and FAA/B7.1 therapy provided complete protection. Anti-tumour CTL activity was still very strong 42 days following initial treatment with DMXAA/B7.1, and was stimulated further following the rechallenge with parental tumour cells (Fig. 3b).

INDUSTRIAL APPLICABILITY

Thus, in accordance with the present invention, the applicants have provided a method of cancer therapy which represents a significant advance over previous approaches in terms of eradication of advanced or large tumours. The advance represented by the present invention is particularly remarkable where the immunotherapeutic agent is administered in appropriate period of time prior to administration of the tumour growth restricting agent. This results in complete eradication of large tumour burdens and the generation of a potent anti-tumour systemic immunity.

Those persons skilled in the art will appreciate that the above description is provided by way of example only and that the present invention is not limited thereto.

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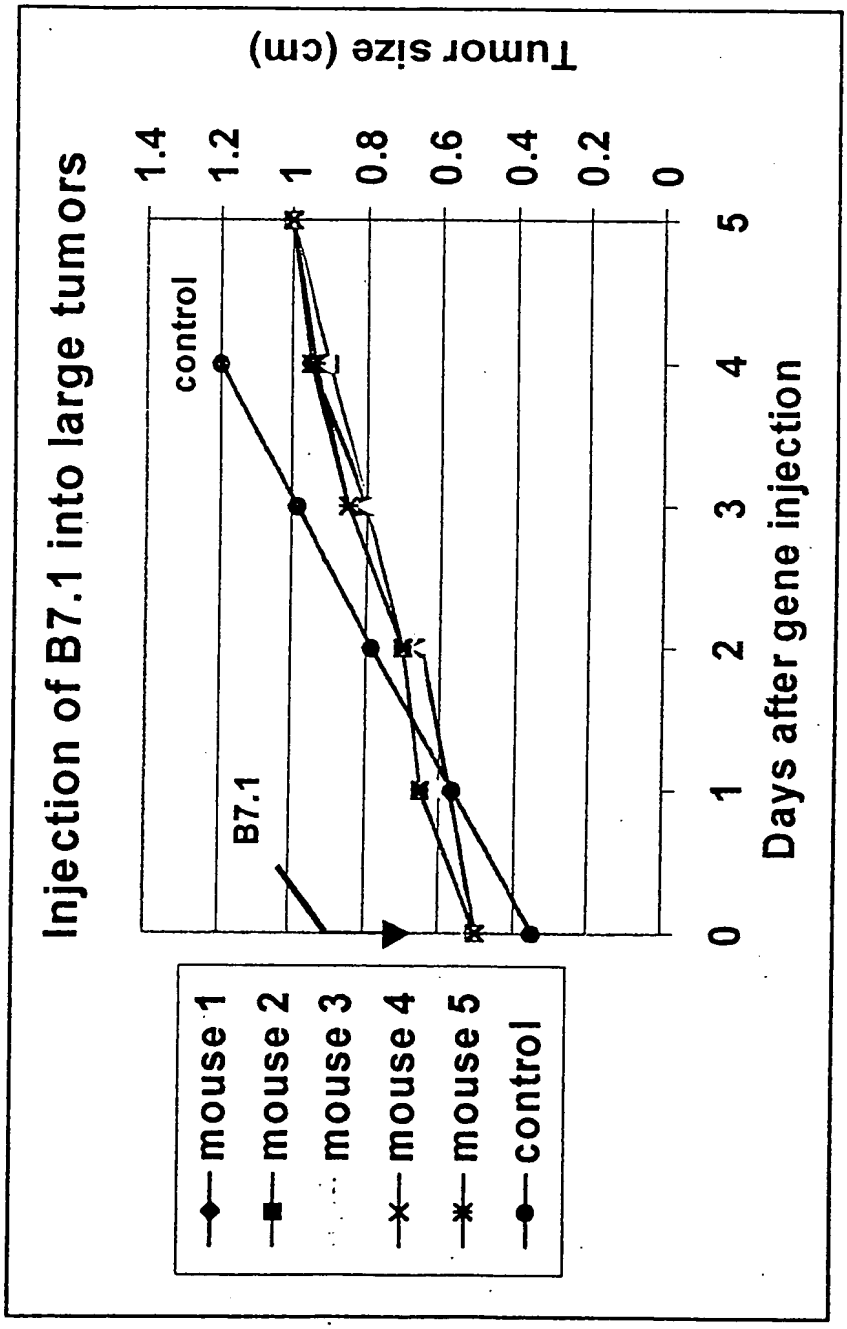
ATTORNEYS FOR THE APPLICANT

FIGURE 1


Injection of B7.1 into large tumors
Tumor size (cm)
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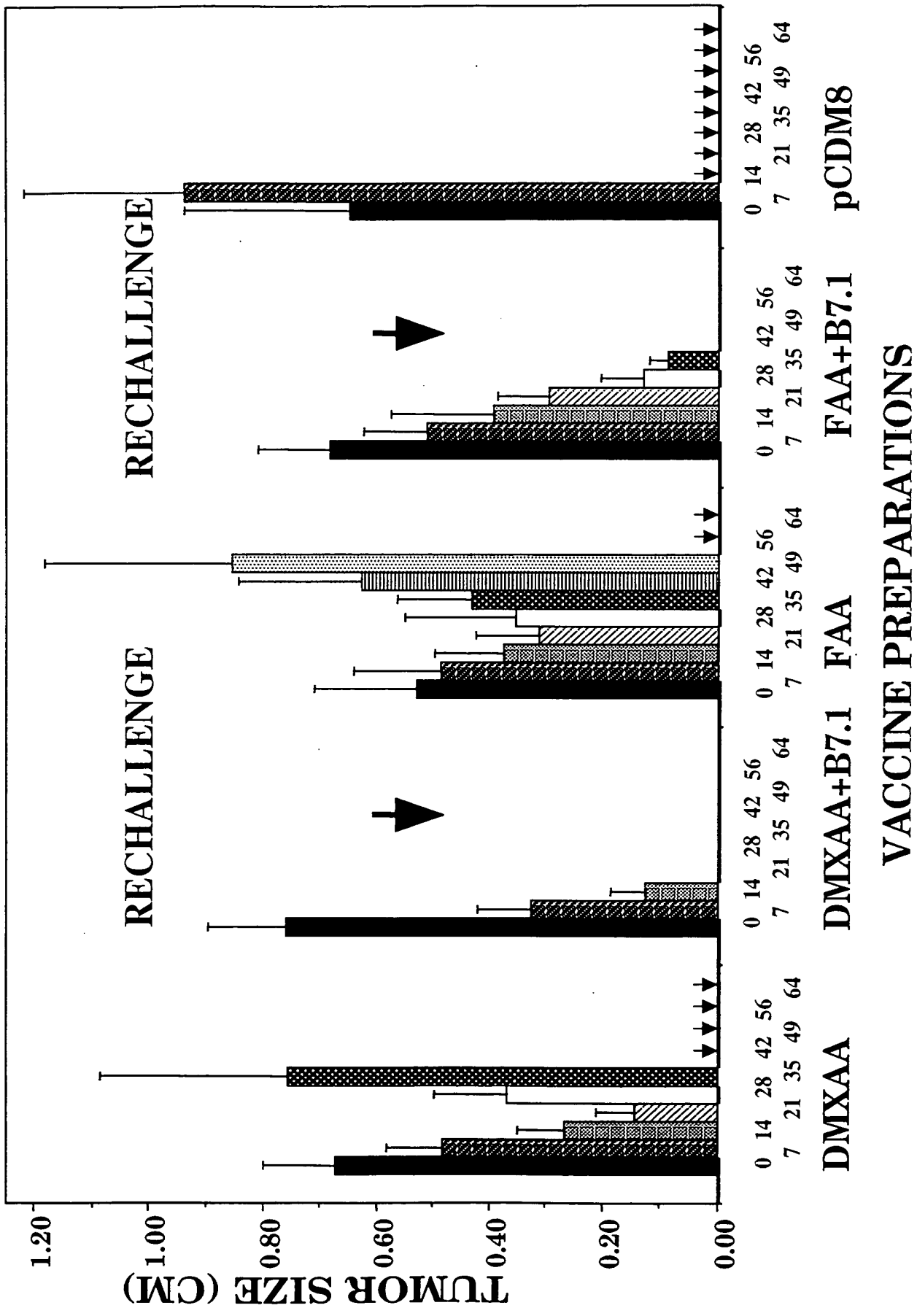
1.2
1
0.8
0.6
0.4
0.2
0
control
B7.1
0
1
2
3
4
5
Days after gene injection
mouse 1
mouse 2
mouse 3
mouse 4
mouse 5
control

FIGURE 2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0.20
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RECHALLENGE
RECHALLENGE
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DMXAA
DMXAA+B7.1
FAA
FAA+B7.1
pCDM8
VACCINE PREPARATIONS

FIGURE 3A

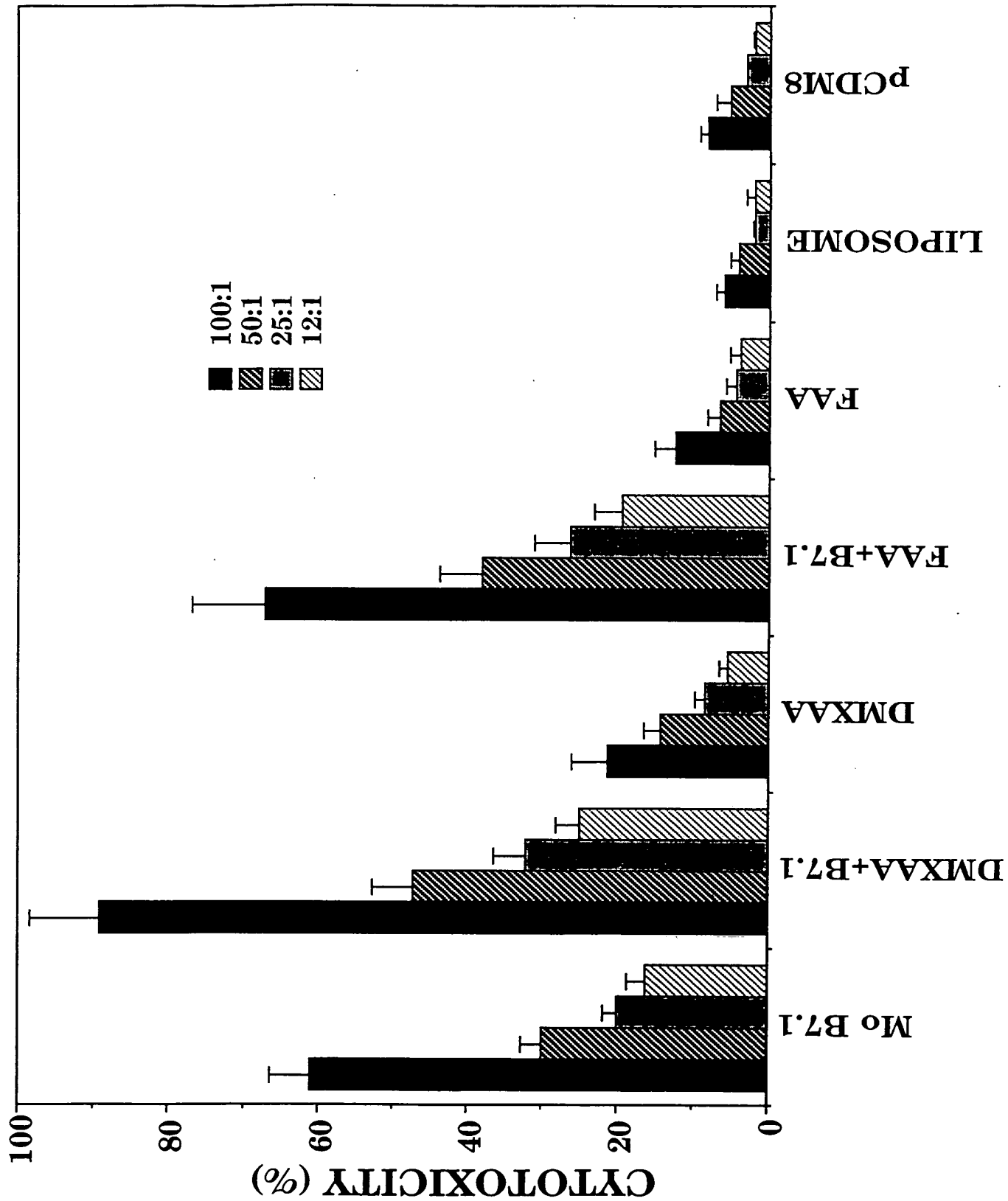
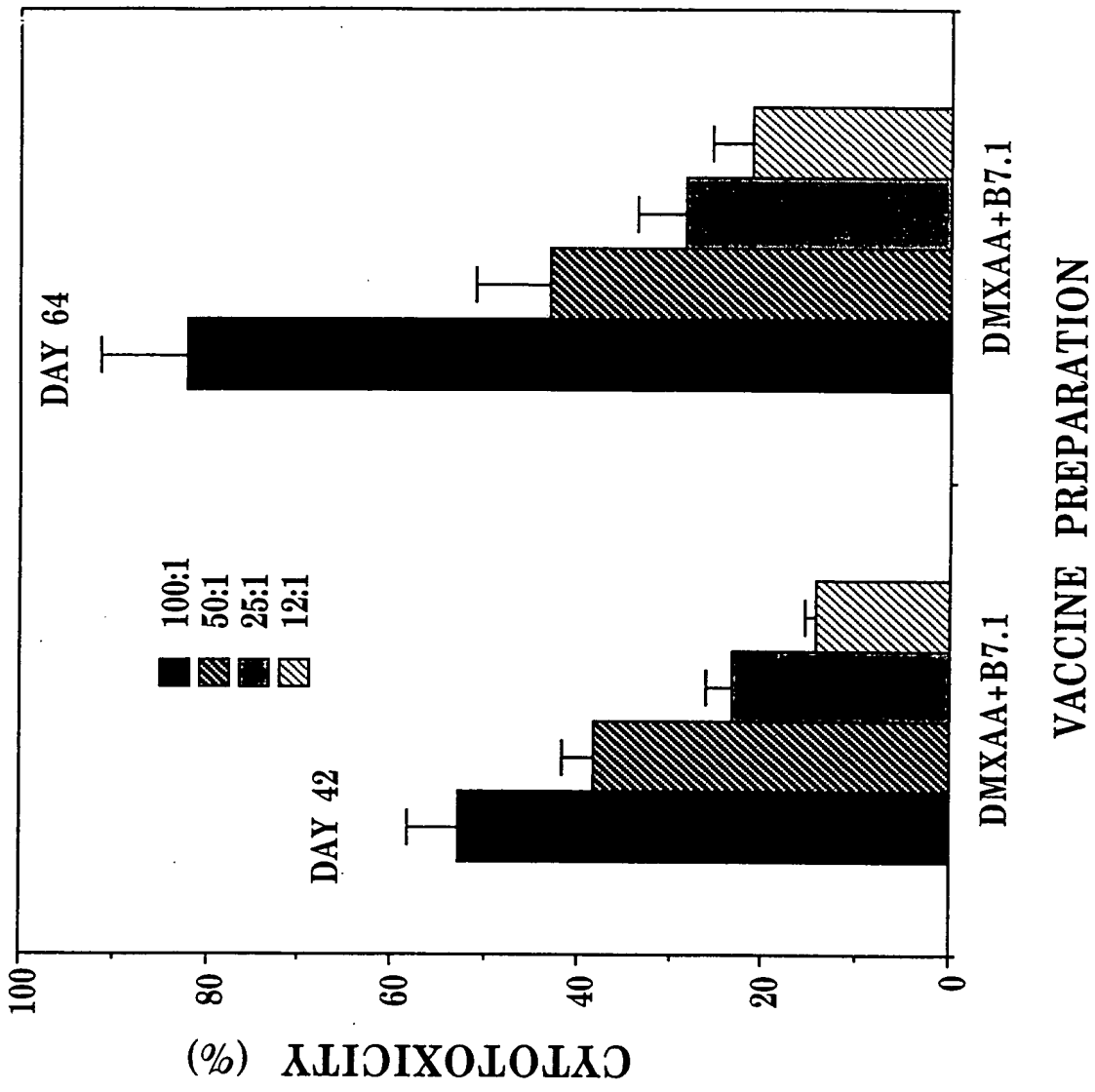

CYTOTOXICITY (%)
0
20
40
60
80
100
100:1
50:1
25:1
12:1
Mo B7.1
DMXAA+B7.1
DMXAA
FAA+B7.1
FAA
LIPOSOME
pCDM8

FIGURE 3B


CYTOTOXICITY (%)
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80
60
40
20
0
DAY 64
DAY 42
100:1
50:1
25:1
12:1
DMXAA+B7.1
DMXAA+B7.1
VACCINE PREPARATION

FIGURE 1

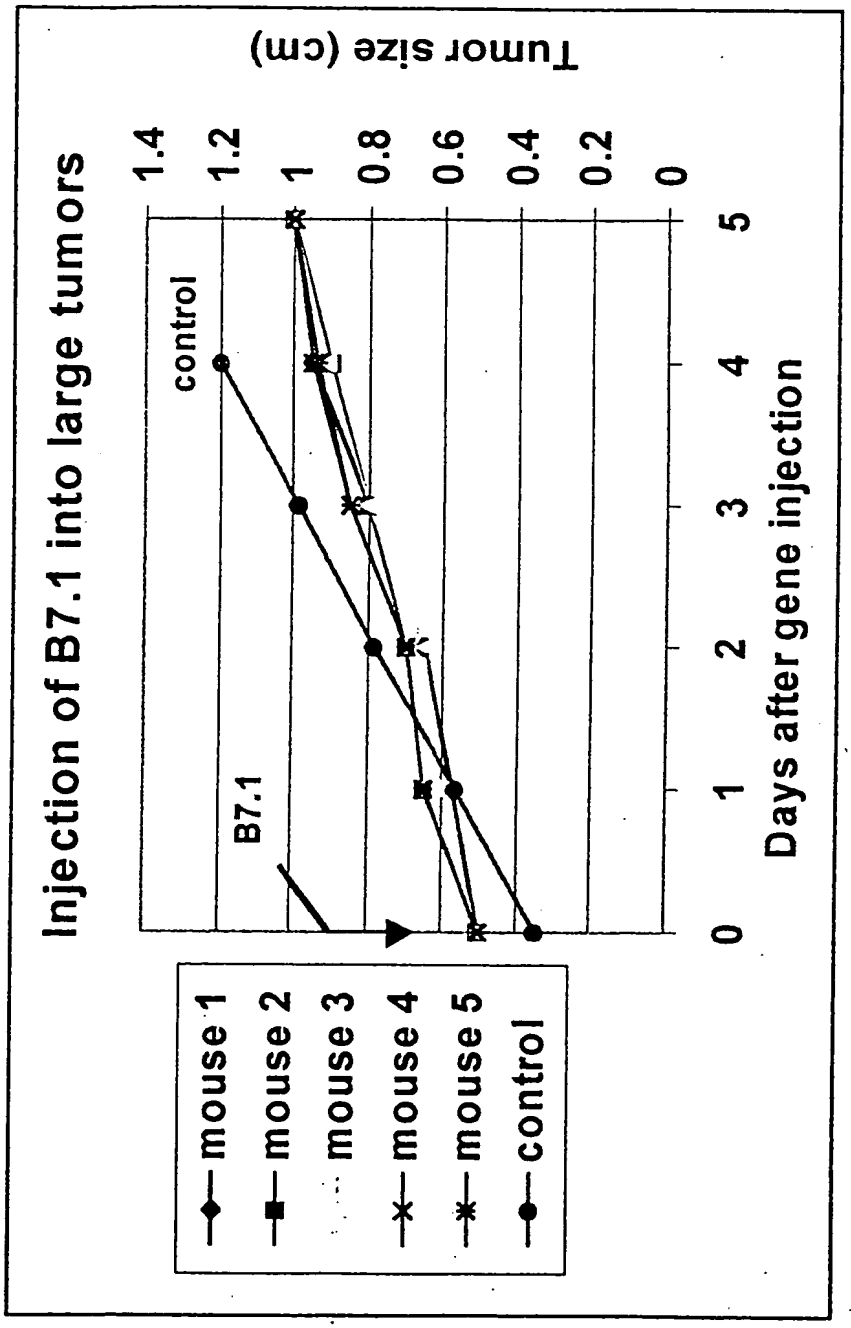
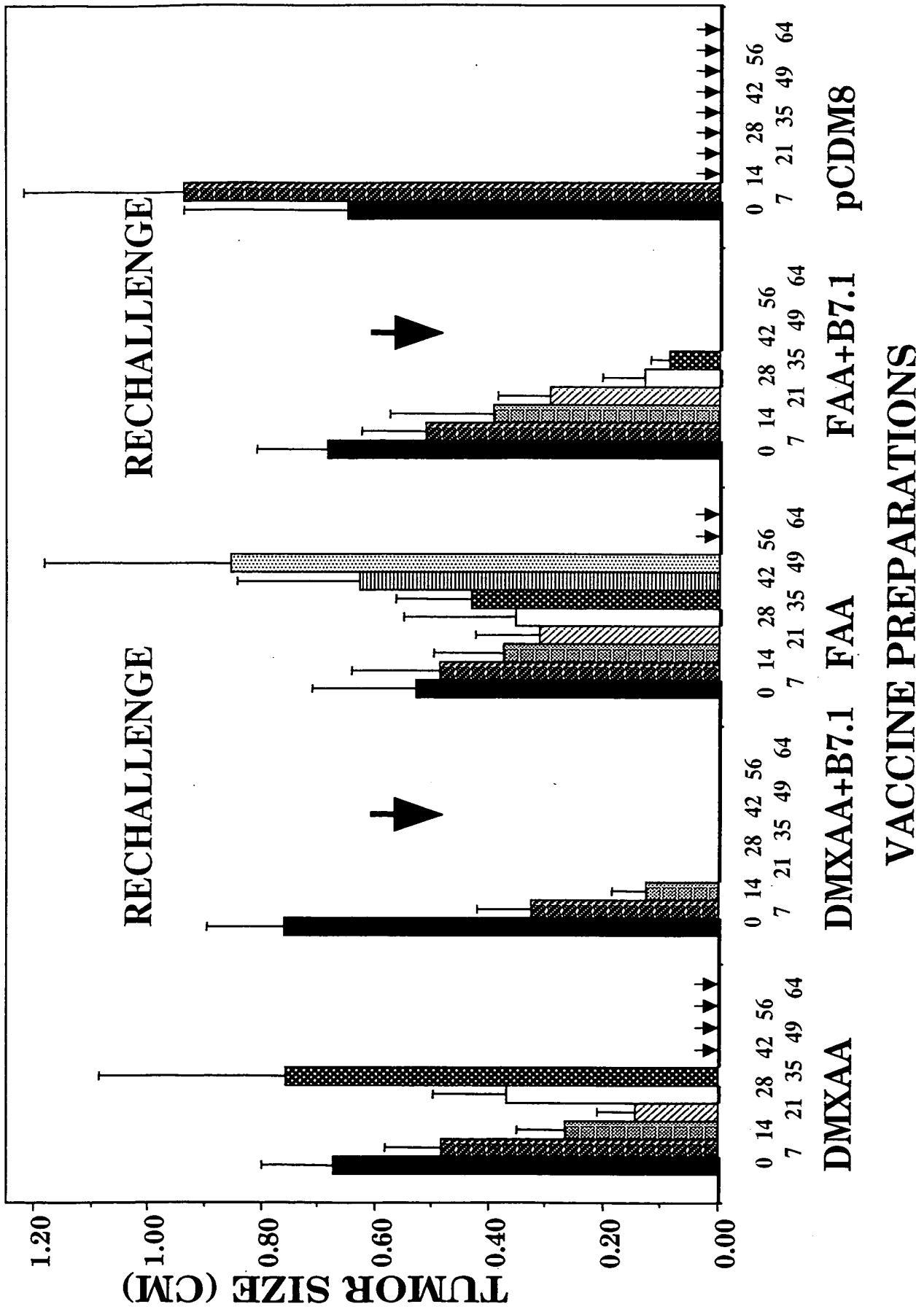

Injection of B7.1 into large tumors
Tumor size (cm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
control
B7.1
0
1
2
3
4
5
Days after gene injection
mouse 1
mouse 2
mouse 3
mouse 4
mouse 5
control

FIGURE 2


TUMOR SIZE (CM)
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1.00
0.80
0.60
0.40
0.20
0.00
RECHALLENGE
RECHALLENGE
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DMXAA
DMXAA+B7.1
FAA
FAA+B7.1
pCDM8
VACCINE PREPARATIONS

FIGURE 3A

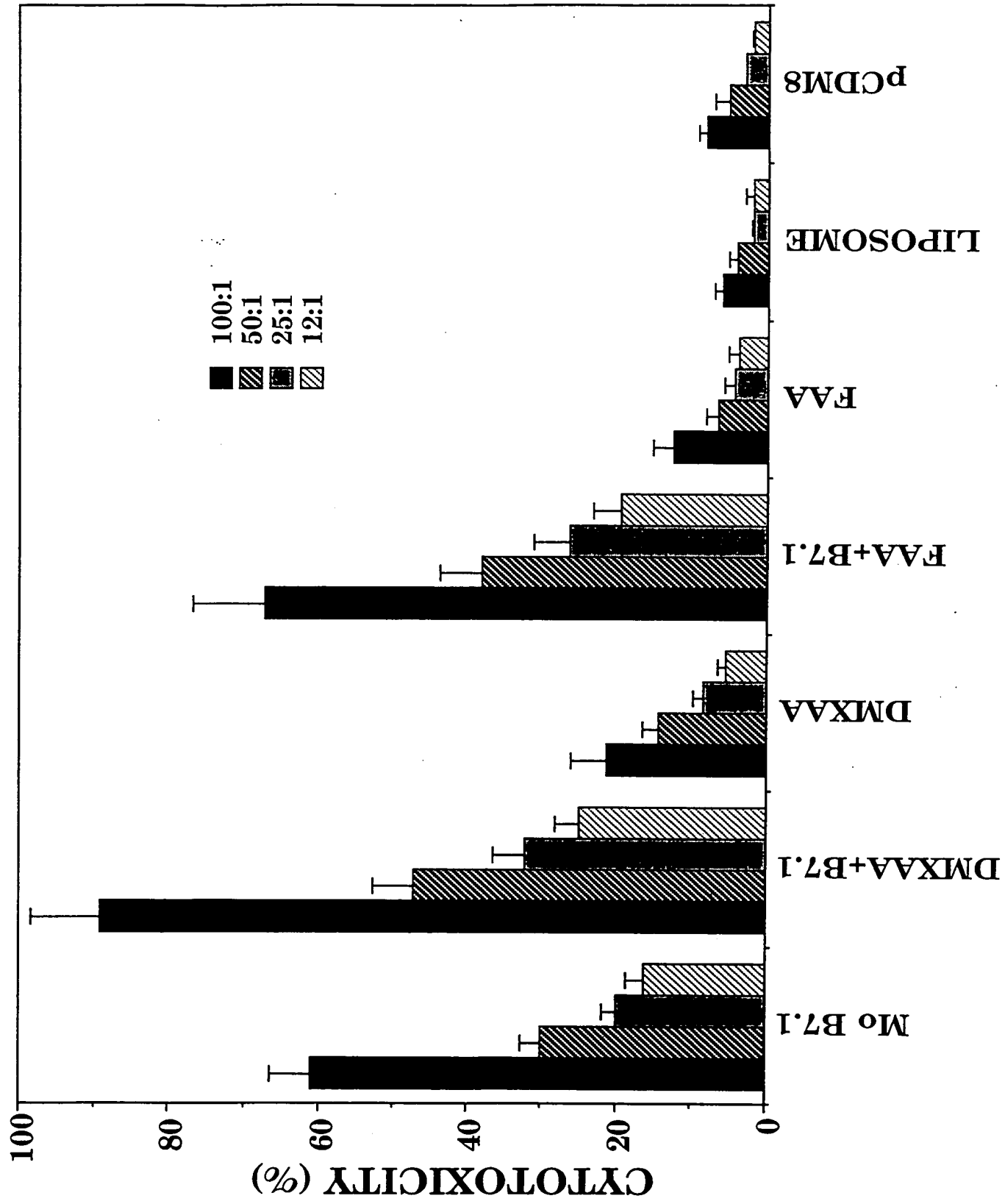
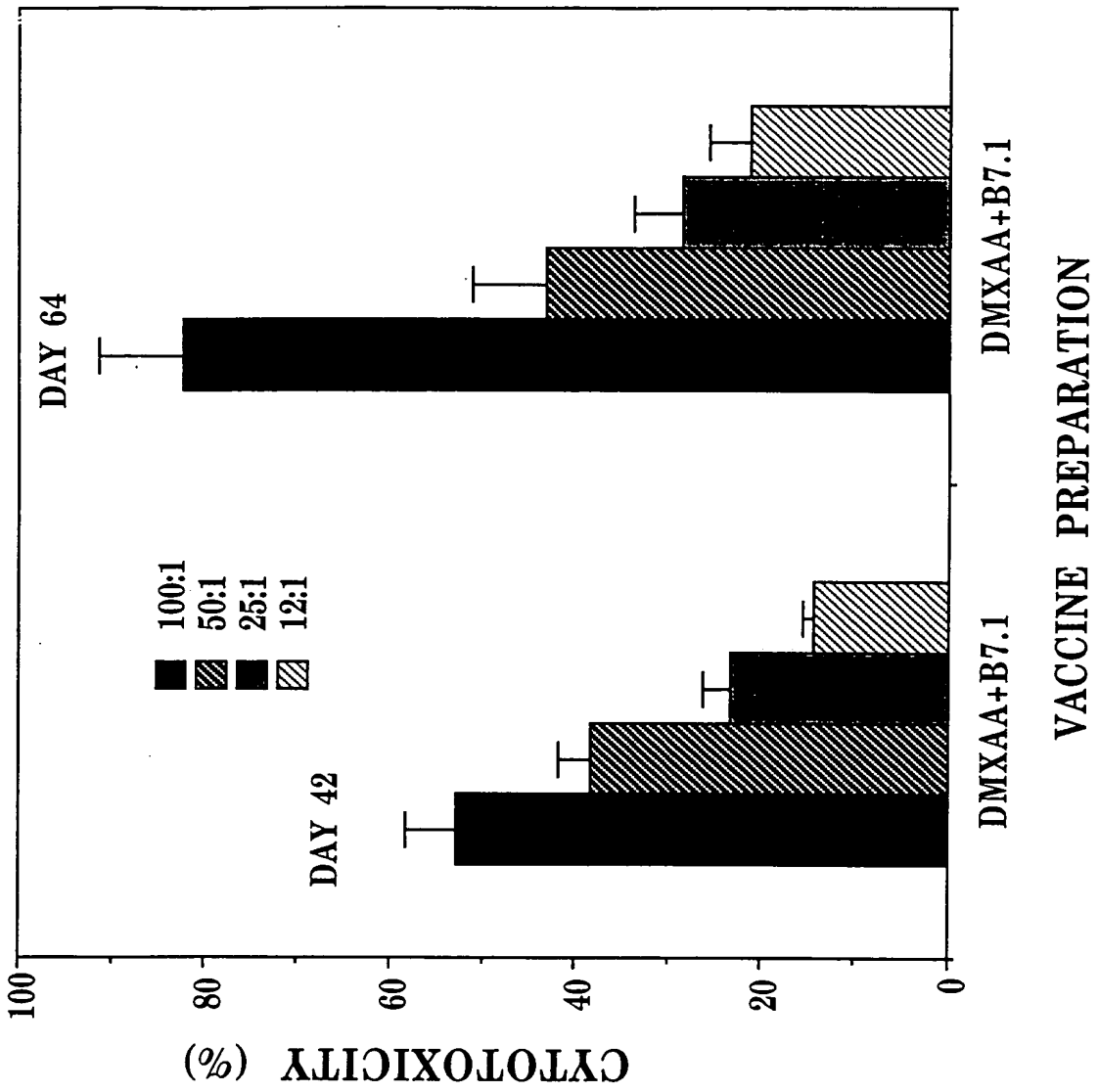

CYTOTOXICITY (%)
0
20
40
60
80
100
100:1
50:1
25:1
12:1
Mo B7.1
DMXAA+B7.1
DMXAA
FAA+B7.1
FAA
LIPOSOME
pCDM8

FIGURE 3B


CYTOTOXICITY (%)
100
80
60
40
20
0
DAY 64
DAY 42
100:1
50:1
25:1
12:1
DMXAA+B7.1
DMXAA+B7.1
VACCINE PREPARATION